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Nuclear Accumulation of p53 Protein Is Mediated by Several Nuclear Localization Signals and Plays a Role in Tumorigenesis

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The basic carboxy terminus of p53 plays an important role in directing the protein into the nuclear compartment. The C terminus of the p53 molecule contains a cluster of several nuclear localization signals (NLSs) that mediate the migration of the protein into the cell nucleus. NLSI, the most active domain, is highly conserved in genetically diverged species and shares perfect homology with consensus NLS sequences found in other nuclear proteins. The other two NLSs, II and III, appear to be less effective and less conserved. Although nuclear localization is dictated primarily by the NLSs inherent in the primary amino acid sequence, the actual nuclear homing can be modified by interactions with other proteins expressed in the cell. Comparison between wild-type p53 and naturally occurring mutant p53 showed that both protein categories could migrate into the nucleus of rat primary embryonic fibroblasts by essentially similar mechanisms. Nuclear localization of both proteins was totally dependent on the existence of functional NLS domains. In COS cells, however, we found that NLS-deprived wild-type p53 molecules could migrate into the nucleus by complexing with another nuclear protein, simian virus 40 large-T antigen. Wild-type and mutant p53 proteins differentially complexed with viral or cellular proteins, which may significantly affect the ultimate compartmentalization of p53 in the cell; this finding suggests that the actual subcellular compartmentalization of proteins may differ in various cell type milieux and may largely be affected by the ability of these proteins to complex with other proteins expressed in the cell. Experiments designed to test the physiological significance of p53 subcellular localization indicated that nuclear localization of mutant p53 is essential for this protein to enhance the process of malignant transformation of partially transformed cells, suggesting that p53 functions within the cell nucleus.

The gene encoding p53 nuclear protein, which has been shown to act as a dominant oncogene (12, 14, 23, 40, 45, 56, 57), was recently reported to function as an antioncogene (13, 15, 29). Using several experimental approaches, it had initially been found that p53 overproduction enhanced the malignant process. However, recent findings suggest that the mutant p53 gene may enhance the malignant process, whereas the wild-type p53 gene functions as an antioncogene.

The hypothesis that the wild-type p53 gene functions as a tumor suppressor gene was initially deduced from the observation that the wild-type protein failed to enhance malignant transformation but rather suppressed the transforming activity of other oncogenes. A comparison of the ability of the various p53 proteins to transform primary embryonic cells in cooperation with the *ras* oncogene indicated that mutant p53 induced the appearance of morphologically transformed foci, whereas the wild type did not (12, 16, 22). Finlay et al. (15) showed that wild-type p53 directly suppressed transformation by E1a and *ras* oncogenes. Eliyahu et al. (13) described a similar suppression by wild-type p53 of *myc* and *ras* transformation. Further support for the idea that the p53 gene may function as an antioncogene comes from the observation that in several types of human and mouse primary tumors, the p53 gene was rearranged and its expression was down-regulated (2, 4, 26, 37–39, 51).

Indirect immunofluorescence staining showed that p53 is localized in the nuclear compartment of transformed cells; normal cells, however, show mainly perinuclear distribution of p53 (44). Subcellular distribution of p53 also varies during the cell cycle. Serum-stimulated cells exhibited nuclear staining, whereas growth-arrested cells demonstrated a faint perinuclear distribution (11). Therefore, the subcellular distribution of p53 may affect the activity of the protein and could be related to differences between wild-type and mutant species.

It is expected that the p53 protein, which is synthesized in the cytoplasm, is actively transported into the nucleus, as is the case with other nuclear proteins (7, 10, 18, 24, 25, 35, 43, 47, 53, 58). Several nuclear proteins, such as simian virus 40 (SV40) large T antigen (24, 25) and nucleoplasmin of *Xenopus laevis* (7), contain specific nuclear localization signals (NLSs). The nuclear homing domains in these proteins, consisting of a basic amino acid core surrounded by α-helix-breaking amino acids, are necessary and sufficient for nuclear localization. In the large T antigen, a Pro–Lys–Lys-128–Lys–Arg–Lys–Val sequence, mapping around amino acid 128, was found to account for the nuclear homing of this protein (24, 25). Substitution of Lys with a Thr at position 128 abolished the capacity of the large T antigen to migrate into the nucleus. An analysis of nucleoplasmin revealed a Gly–Gln–Ala–Lys–Lys–Lys–Lys–Leu–Asp–Lys sequence at the C-terminal end of the molecule that was shown to function as an NLS as well (7). Sequence analyses of other nuclear proteins such as the polyomavirus large T antigen (43) and the SV40 VP1 (58) indicated the presence of NLSs that share homology with the specific domain found in the large T antigen (24, 25). Conjugation of polypeptides containing the nuclear homing signal to bacterial β-galactosidase (18) or to chicken serum albumin (8), which would otherwise accumulate in the cytoplasm, resulted in the migration of these fusion proteins into the nucleus upon cytoplasmic microinjection.

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In this study, we investigated the mechanism responsible for nuclear migration of the p53 protein and the functional significance of its subcellular localization. In agreement with previous reports (1, 9), we found that this protein contains a principal NLS, NLSI, which accounts for its nuclear localization. However, a detailed site-directed mutagenesis (SDM) analysis indicated the presence of additional NLSs, all localized at the C terminus of the molecule. Although its nuclear localization is primarily dictated by the presence of NLSs inherent in its structure (1, 9), we report here that the ultimate compartmentalization of the p53 protein is largely affected by the formation of complexes with cellular or viral proteins expressed in the cell. A comparison of wild-type and mutant p53 revealed that while nuclear localization of both proteins in normal primary rat embryo fibroblasts (REF) is totally dominated by functional NLS domains, their subcellular distribution in COS cells may be modified by their different abilities to bind specifically with the expressed large T antigen. We found that NLS-deprived wild-type and mutant p53 do not migrate into the nucleus of primary REF. In COS cells, however, nuclear localization of NLS-deprived wild-type, but not mutant, p53 can be facilitated by formation of a complex with the large T antigen. Furthermore, we show that migration into the nucleus plays an essential role in the transforming activity of the protein and that NLSs are important for mutant p53 protein to enhance tumor development of partially transformed cell, suggesting that p53 functions within the nucleus.

MATERIALS AND METHODS

Cell culture and DNA transfection. COS cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum and 20 mM L-glutamine. For DNA transfection, cells were plated at 1×10^6 to 5×10^6 cells per 10-cm-diameter plate 24 h before transfection. REF were prepared from 14- to 15-day-old Fisher 344 rat embryos and maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Plasmid DNA was transfected by the calcium phosphate precipitation method (17). At 24 to 72 h following transfection, we analyzed p53 expression by immunoprecipitation, immunoblotting, or immunofluorescence as described below.

L12 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2×10^{-5} M β-mercaptoethanol, and 20 mM L-glutamine. L12-derived cell lines were established by electroporation of the p53-derived plasmids in conjunction with plasmid pSV2-gpt as described before (50). The clones were maintained in selectable RPMI 1640 medium containing mycophenolic acid (2 μg/ml), xanthine (150 μg/ml), and hypoxanthine (15 μg/ml). Single-cell clones were isolated, and the number of integrated DNA copies was estimated by Southern blot analysis. p53 protein levels were estimated by Western immunoblot analysis.

Plasmid construction and SDM. The mammalian expression vector used for all p53 cDNAs in these experiments was pSVL (Pharmacia). This vector consists of SV40 late promoter, VP1 intron, and SV40 late polyadenylation signal. The various cDNA clones were inserted into the unique *Bam*HI site of the polylinker. The p53-M8 and p53-M11 constructs consisted of the respective full-length cDNA clones isolated from a λgt10 Meth-A cDNA library (55). The p53-cD clone, isolated from a normal T-cell library, consists of the full-length wild-type p53 and an additional 95-bp containing an SV40 splice acceptor sequence upstream of the coding region, derived from the original pcD-p53 plasmid (3).

Deletion of NLSI from p53-M8 was performed by removing a *Bsp*MI-*Sac*II fragment from the cDNA in the pSVL vector. The protruding ends were filled in with a large fragment of *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories). The deleted DNA was dephosphorylated with calf intestinal phosphatase (Boehringer Mannheim), purified by gel electrophoresis, and ligated to a phosphorylated *Bgl*II linker (New England BioLabs). The ligation mixture was transfected into competent *E. coli* HB101 bacteria, and the resulting plasmids were analyzed by restriction analysis and by sequencing across the newly formed junctions by using the TaqTraq sequencing system (Promega).

For site-specific mutagenesis, the p53 cDNAs were cloned as *Bam*HI fragments into the Bluescript M13+ vector (Stratagene), from which the single-stranded template was prepared. Oligonucleotide-directed SDM was performed essentially according to the Bio-Rad MUTA-GENE kit protocol, using single-stranded DNA which was produced in *E. coli* CJ236 as a template. The oligonucleotides were produced by the Chemical Services Department of the Weizmann Institute of Science and were purified to size homogeneity by fractionation on a polyacrylamide gel. The oligonucleotides used were GCGTTTTCTGTTTTGGTGA (used to modify NLSI), corresponding to bases 943 to 961 (the predicted modification is from T to G at position 952) and ACTTCTG GTGCTTCCCGGT (used to modify NLSII), corresponding to bases 1099 to 1117 (the predicted modification is from T to G at position 1108). The extension reaction was carried out by using T4 DNA polymerase and T4 gene 32 product (Boehringer Mannheim), transfected into *E. coli* TG1 competent bacteria. Mutated plasmids were analyzed by DNA sequencing, using the TaqTraq sequencing system (Promega). The mutated p53 cDNAs were subcloned into the pSVL mammalian expression vector. Double mutants were created by replacing a *Sac*II-*Bam*HI DNA fragment, downstream of the mutated NLSI construct, with a corresponding fragment from a mutated NLSII-derived DNA. Sequence analysis was performed to verify these chimeras.

Antibodies. Monoclonal anti-p53 antibodies were obtained from the established hybridoma cell lines PAb-242, PAb-246, PAb-248 (59), and PAb-421 (19). Monoclonal anti-SV40 large T antigen antibodies were obtained from the established hybridoma cell line PAb-419 (18). Supernatants of confluent hybridoma cell cultures were filtered through a 0.45-μm-pore-size nitrocellulose membrane and used without further purification.

Affinity-purified goat anti-mouse immunoglobulin G antibodies (Bio Yeda, Rehovot, Israel) were labeled with rhodamine B (6) to a rhodamine/protein molar ratio in the conjugates of about 3. These antibodies were used for indirect immunofluorescence staining. Antibodies were stored at 4°C in the presence of 1.5 mM sodium azide.

Indirect immunofluorescence staining. Cells were plated on sterile cover slides and transfected as described above. At 24 to 36 h after transfection, the cells were fixed for 15 min with 3% formaldehyde in phosphate-buffered saline (PBS). The fixed cells were washed twice with PBS, permeabilized for 5 min in acetone at −10°C, and washed twice with PBS.

For indirect immunofluorescence, slides were incubated for 30 min with the appropriate antibody. Slides were washed for 15 min with PBS at room temperature and incubated for another 30 minutes with a rhodamine-conjugated goat anti-mouse immunoglobulin G antibody. Finally,

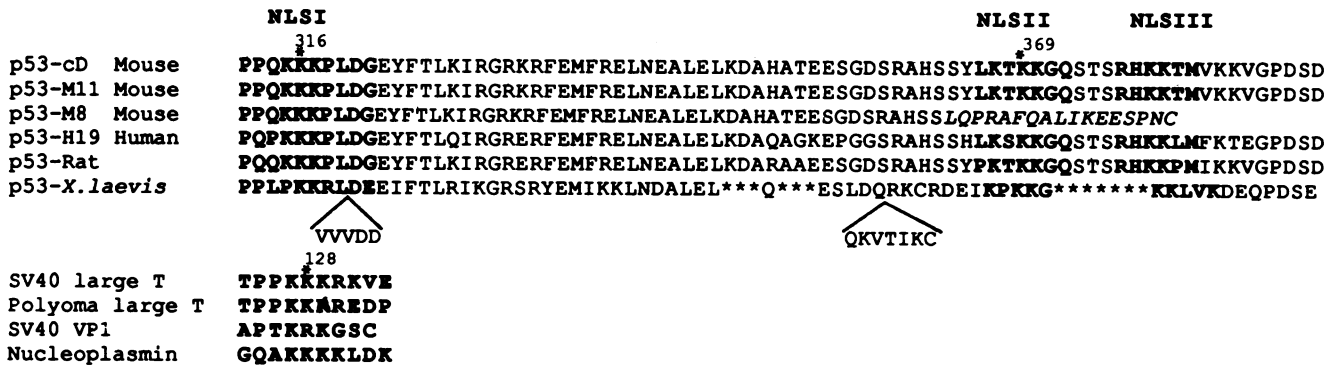

FIG. 1. Sequence comparison of various NLSs contained in the carboxy termini of p53 proteins. The boldface letters indicate the predicted NLS domains in p53 compared with the NLS domains of other nuclear proteins. Asterisks indicate missing amino acids, and inserts indicate additional amino acids in the *X. laevis* p53 protein.

the slides were washed extensively in PBS, rinsed with water, and mounted on objective microscope slides with Gelvatol (Monsanto).

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were labeled for 2 h at 37°C in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated dialyzed fetal calf serum and 0.125 mCi of [^{35}S]methionine (Amersham). Cells were lysed in RIPA buffer and precleared with fixed *Staphylococcus aureus*. Equal amounts of trichloroacetic acid-insoluble radioactive material were reacted with the specific antibodies for 2 to 12 h at 4°C. The immune complexes were precipitated with fixed *S. aureus* (27) and washed four times with RIPA buffer. Polyacrylamide gel electrophoresis was performed as described previously (28).

For immunoblotting, cells were lysed in sample buffer and subjected to polyacrylamide gel electrophoresis as described above. The fractionated proteins were electrotransferred to nitrocellulose membranes, and the proteins were detected by using the ProtoBlot Western Blot AP system (Promega).

Tumorigenicity assay in mice. L12-derived clones from the earliest passage available, expressing the p53-M8 or the p53-M8$^{NLSI^-}$ cDNA, were grown in RPMI 1640 medium as described above without the selective drugs for 48 h prior to injection. The cells were washed and resuspended in PBS, and 5×10^6 cells were injected subcutaneously into individual syngeneic male C57L/J mice. The mice were monitored for tumor development and were graded from 0 to 4 according to the relative size of the tumor and overall clinical status; 0, no detectable tumor; 1, a visible tumor smaller than 0.5 cm in diameter; 2, a tumor larger than 0.5 cm in diameter and no obvious paralysis; 3, a tumor larger than 0.5 cm in diameter and partial or complete paralysis; 4, death. At each survey, the individual scores were summed. The sum was divided by the maximal possible score (which represents 100% death) and multiplied by 100.

RESULTS

Putative NLSs contained in the p53 protein. Sequence analysis of murine p53 suggests the existence of three putative NLSs, all clustered at the C terminus. The first domain, NLSI, is the peptide sequence Pro–Pro–Gln–Lys–Lys–Lys–Pro–Leu–Asp–Gly, positioned between amino acids 313 and 322. This sequence has the essential features of a consensus NLS. The three Lys residues form the basic core that is surrounded by α-helix-breaking Pro residues. This domain is almost identical in human (20, 33), murine (3, 60), and rat (48) p53 and is highly conserved in *X. laevis* p53 (49), suggesting that it is functionally important. Two other putative NLS sequences, NLSII and NLSIII, are less conserved and share lower homology with the NLS found in SV40 large T antigen. Figure 1 compares the sequences of different p53 NLSs found in the mouse, human, rat, and *X. laevis* genomes with previously published NLS sequences. It should be noted that the putative NLSI of mutant and wild-type mouse p53 is identical in proteins encoded by p53-M11, p53-M8 and p53-cD. Sequence variations between these mouse p53 proteins lie outside this NLS domain (3). p53-M11 and p53-cD also share perfect homology in the rest of the C-terminal end, downstream of NLSI, but p53-M8 lacks the putative NLSII and NLSIII.

Transient expression of p53 cDNA clones in COS cells. To identify the functional NLS of p53, our strategy was to delete or mutate the sequences coding for the putative NLSs of p53 cDNA clones and study alterations in the subcellular localization of the encoded p53 proteins. The assay used in these experiments consisted in transfecting modified p53 cDNA clones, constructed in the pSVL expression vector, into COS cells and analyzing transient expression of p53 protein. Subcellular localization of the protein was monitored by indirect immunofluorescence staining of fixed cells. We used species-specific anti-p53 monoclonal antibodies to distinguish between the endogenous monkey p53 protein expressed in COS cells and the exogenous transfected murine p53 protein.

First, we compared the subcellular localizations of p53 proteins encoded by various p53 cDNA clones isolated from nontransformed and transformed cells. Of interest were p53-M8 and p53-M11, isolated from Meth-A-transformed fibroblasts, representing mutant p53, and p53-cD, isolated from nontransformed stimulated T-cells, representing wild-type p53 (3, 55). p53-M11 and p53-M8 differ at the 3′ end as a result of alternative splicing (3). Whereas p53-M11 and p53-cD code for proteins that carry all three putative NLS sequences, p53-M8 codes for a protein that lacks NLSII and NLSIII but retains NLSI.

The various p53 constructs and the parental pSVL vector were transfected into COS cells; 36 to 48 h later, the cells were either metabolically labeled or fixed for indirect immunofluorescence staining. p53 proteins of metabolically labeled total-cell extracts were immunoprecipitated with the following monoclonal antibodies: PAb-242, PAb-246, and PAb-248, which are murine specific; PAb-421, which recognizes the endogenous monkey as well as the transfected p53

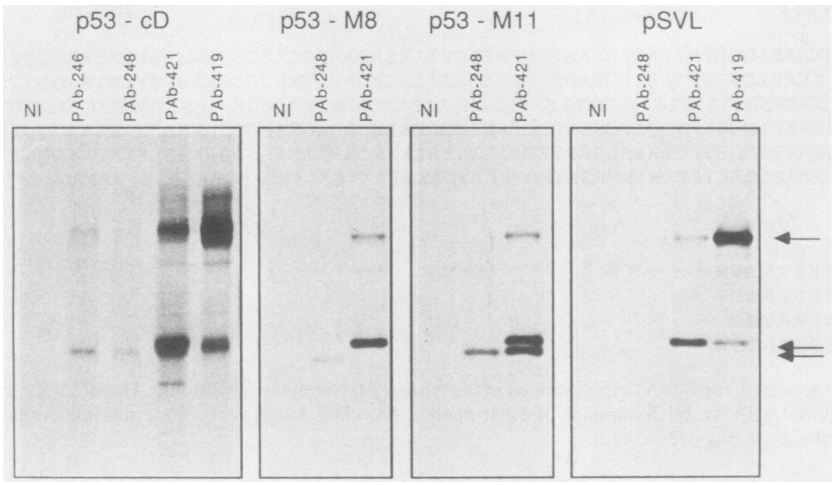

FIG. 2. Transient expression of murine p53 cDNA clones in COS cells. COS cells were transfected with the various p53 cDNA clones constructed in the pSVL vector. Thirty-six hours after transfection, cells were labeled with [^{35}S]methionine for 2 h. Equal amounts of trichloroacetic acid-insoluble labeled cell lysates were immunoprecipitated with monoclonal antibodies, and the immune complexes were fractionated on a 10% polyacrylamide-sodium dodecyl sulfate gel, processed for autoradiography, and exposed to X-ray film. Lanes: NI, nonimmune supernatant; PAb-246, PAb-248, and PAb-421; anti-p53 monoclonal antibodies; PAb-419, anti-SV40 large T antigen. Arrows designate the SV40 large T antigen (top), endogenous monkey p53 (middle), and transfected murine p53 (bottom).

murine protein; and PAb-419, which is a monoclonal antibody directed against SV40 large T antigen. All murine p53 cDNAs coded for proteins of the expected size that retained their specific antigenic determinants, and the protein encoded by p53-M11 was similar in size to that encoded by wild-type p53-cD (Fig. 2). Both proteins were recognized by all of the expected anti-p53 monoclonal antibodies. As observed before (55), the protein encoded by p53-M8 was smaller and lacked the PAb-421 antigenic determinant. The endogenous p53 product was detected by PAb-421 only. In agreement with previous reports, we found that while the wild-type p53 protein encoded by p53-cD formed a complex with the large T antigen, the mutant murine proteins did not form such a complex (52). We found that in COS cells, the large T antigen consistently formed a complex with the endogenous monkey p53, regardless of transfection of the various p53 constructs.

To determine the subcellular localization of p53 proteins encoded by the transfected cDNAs, we performed indirect immunofluorescence staining, using the murine p53-specific antibodies PAb-242, PAb-246, and PAb-248. While 100% of cells expressing the wild-type p53 exhibited an exclusive nuclear staining, only 85% of cells expressing the mutant protein encoded by p53-M11 showed a similar strictly nuclear staining pattern (Fig. 3F and G; Table 1). The remaining 15% of the p53-M11-transfected cells demonstrated a weak cytoplasmic staining in addition to nuclear staining. This difference in the subcellular localization of wild-type and mutant p53 may be attributable to their differential binding to the SV40 large T antigen expressed in the COS cells (52). In COS cells transfected with the p53-M8 constructs, 100% of the transfected cells exhibited a strong nuclear staining, which was consistently accompanied by a weak cytoplasmic staining (Fig. 3D and E). The fact that p53-M8, which retains only NLSI, exhibited the same nuclear staining as was observed in the presence of all three putative p53 NLS sequences strongly suggests that NLSI is an effective nuclear signal of the p53 molecule. The p53-M8-encoded protein, which carries only NLSI, reproducibly exhibited simultaneous nuclear and cytoplasmic staining, which suggests that other NLS sequences may play a role in the nuclear homing of the full-length p53 protein.

Deletion of NLSI in the protein encoded by p53-M8 cDNA clone. To test the assumption that NLSI is indeed the principal sequence responsible for the nuclear homing of the p53 molecule, we focused on clone p53-M8, which contains a single site, NLSI. In the next experiment, we deleted the region containing the NLSI sequence and monitored modulations in the subcellular distribution of the encoded p53 molecules. To that end, we substituted a 68-nucleotide *Bsp*MI-*Sac*II fragment at the 3′ end of the cDNA with a *Bgl*II polylinker. This procedure exchanged 13 amino acids of the original protein sequence with 3 novel amino acids, giving rise to a shorter in-frame p53 protein that lacked the NLSI sequences (Fig. 4). The structure of the plasmid, p53-M8$^{\Delta NLSI}$, generated by this manipulation was confirmed by sequencing across the novel junction formed. This deleted construct was transfected into COS cells, and subcellular localization of the encoded protein was determined. While the intact p53-M8 protein exhibited mostly nuclear staining accompanied by a weak cytoplasmic signal (Fig. 4A), the deleted protein encoded by p53-M8$^{\Delta NLSI}$ was localized mainly in the cytoplasmic compartment (Fig. 4B). This staining pattern confirms the assumption that the 13 amino acids containing the putative NLSI sequence (around Lys-316) are indeed essential for the nuclear localization of p53-M8.

SDM in NLSI of p53 cDNA clones. To better define the specific element responsible for the nuclear homing of p53, we next modified the sequence of the putative NLSI by SDM. On the basis of studies with the large T antigen, it was expected that modification of the central Lys to a Thr in NLSI would significantly interfere with the nuclear homing ability of the p53 protein. This modification was expected to perturb the basic core created by the three contiguous lysines. We therefore modified the adenine at position 952 to a cytidine and thus substituted the central Lys (amino acid 316) with Thr in the two mutant p53 cDNA clones, p53-M8 and p53-M11, generating clones p53-M8$^{NLSI^-}$ and p53-M11$^{NLSI^-}$, respectively. Western blot analysis of the mu-

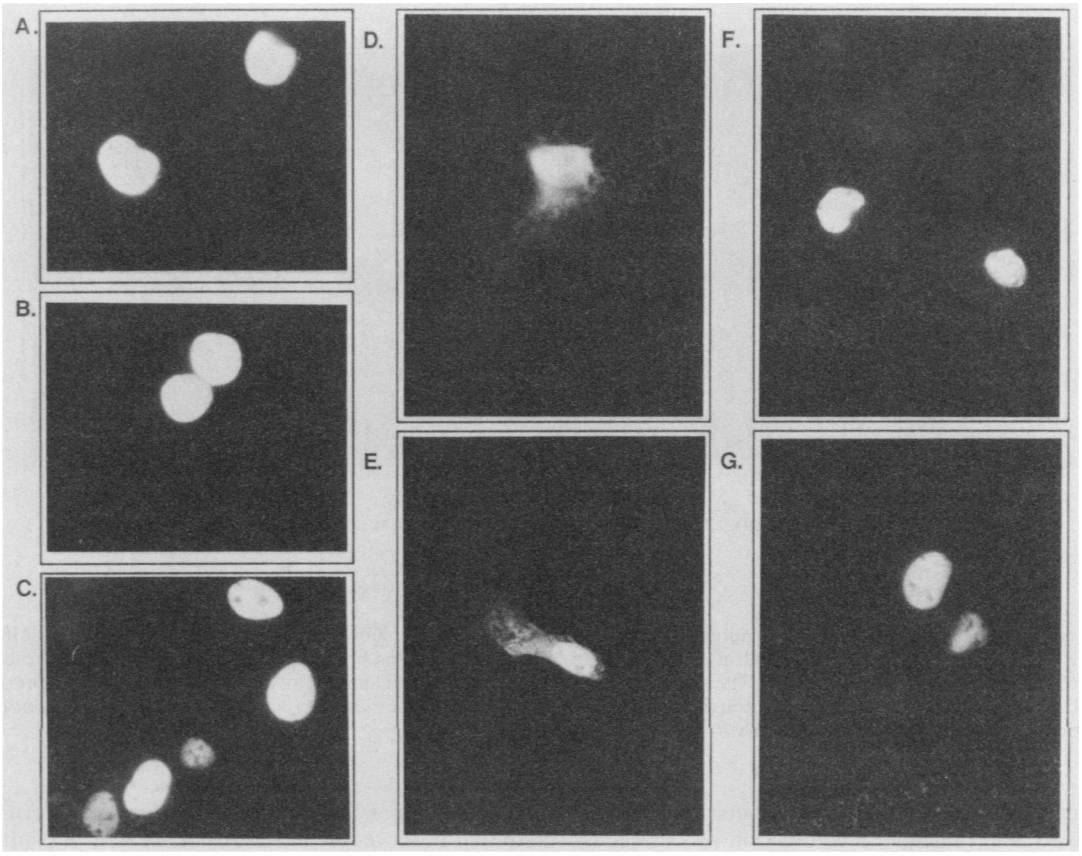

FIG. 3. Subcellular localization of p53 proteins encoded by various cDNA clones. COS cells were transfected with p53 cDNA clones constructed in the pSVL vector. At 24 to 36 h after transfection, cells were fixed and processed for indirect immunofluorescence staining with different p53-specific monoclonal antibodies. (A to C) COS cells transfected with p53-cD and stained with PAb-242 (A), PAb-248 (B), and PAb-246 (C); (D and E) COS cells transfected with p53-M8 and stained with PAb-242 (D) and PAb-248 (E); (F and G) COS cells transfected with p53-M11 and stained with PAb-242 (F) and PAb-248 (G). In all cases, the fluorescent second antibody was a rhodamine-conjugated goat anti-mouse antibody.

tated p53 products indicated that both p53-M8$^{NLSI^-}$ and p53-M11$^{NLSI^-}$ maintained the original size and coded for a p53 protein expressing the respective antigenic epitopes (Fig. 5).

We found that transfection of the SDM-mutated M8 cDNA, p53-M8$^{NLSI^-}$, into COS cells yielded a protein that accumulated in the cytoplasm of 100% of the transfected cells (Fig. 6G and H; Table 1). This finding agreed with the results obtained with the p53-M8$^{\Delta NLSI^-}$ construct and implied that NLSI is located around Lys-316 of p53-M8 and that this domain dictates the nuclear migration of the p53 protein. The immunofluorescence staining of COS cells expressing p53-M11$^{NLSI^-}$ showed an equal distribution of p53 protein between the nucleus and the cytoplasm in 100% of the transfected cells (Fig. 6B; Table 1). That a mutation in NLSI of p53-M8 completely abolished the nuclear accumulation of the protein, whereas the SDM mutation in p53-M11 reduced nuclear localization by only 50%, supports the notion that the full-length p53 protein carries an additional nuclear localization activity.

SDM in NLSII of p53 cDNA clones. In NLSII, the basic core formed by the lysine residues is interrupted by threonine. From a comparison with other known NLS sequences, it was predicted that NLSII would be less effective than

TABLE 1. Subcellular distribution of the various p53 encoded proteins in transfected cells

cDNA	SDM mutation	T-antigen binding	Localization in COS	Localization in REF
p53-M8	None	−	Strong nuclear and weak cytoplasmic	ND[a]
p53-M8$^{NLSI^-}$	NLSI	−	Cytoplasmic	Cytoplasmic
p53-M11	None	−	85% nuclear, 15% strong nuclear and weak cytoplasmic	As in COS
p53-M11$^{NLSI^-}$	NLSI	−	Nuclear and cytoplasmic with equal intensity	ND
p53-M11$^{NLSII^-}$	NLSII	−	50% nuclear, 50% strong nuclear and weak cytoplasmic	ND
p53-M11$^{NLSI^-,NLSII^-}$	NLSI, NLSII	−	61% cytoplasmic, 39% strong cytoplasmic and weak nuclear	As in COS
p53-cD	None	+	Nuclear	Nuclear
p53-cD$^{NLSI^-}$	NLSI	+	76% nuclear, 24% nuclear and weak cytoplasmic	ND
p53-cD$^{NLSI^-,NLSII^-}$	NLSI, NLSII	+	60% nuclear, 40% nuclear and cytoplasmic at equal intensity	Cytoplasmic

[a] ND, Not done.

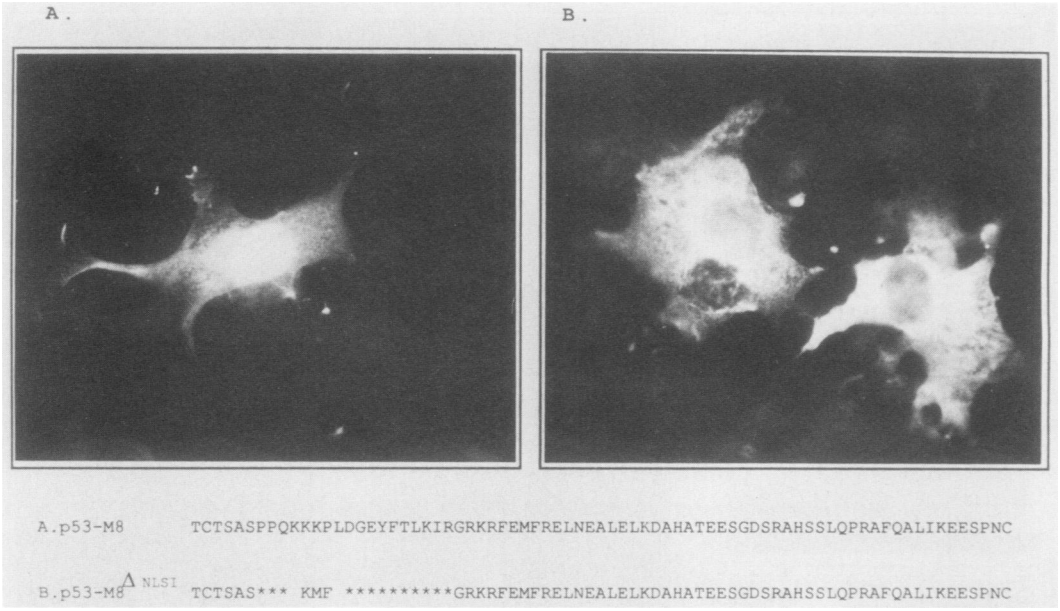

FIG. 4. Alterations in the subcellular localization of NLS-deprived p53 protein. COS cells were transfected with p53-M8 (A) or with p53-M8$^{\Delta NLSI}$ (B) in the pSVL vector. At 24 to 36 h after transfection, cells were fixed and processed for indirect immunofluorescence staining with the monoclonal anti-p53 antibody PAb-248. The amino acid sequences at the bottom are the respective primary sequences of the original and the deleted proteins from amino acid 306 through the end of the protein. Asterisks denote the deleted amino acids. Between these are the three additional amino acids derived from the *Bgl*II inserted linker.

NLSI in directing p53 into the nucleus. A mutation in NLSII was expected to affect nuclear accumulation less markedly than would a mutated NLSI. We mutated the p53-M11 cDNA by substituting the adenine at position 1108 with cytidine, causing the replacement of Lys-369 with a Thr, thus converting Lys–Thr–Lys-369–Lys into Lys–Thr–Thr–Lys. COS cells transfected with this p53-M11$^{NLSII^-}$ cDNA exhibited a strong nuclear staining; however, more than 50% of the cells expressed, in addition, a clear cytoplasmic staining (Fig. 6C; Table 1). This staining pattern was similar to that observed in cells transfected with the intact p53-M8, which lacked NLSII and III (Fig. 3D and E). Since only about 50% of the p53-M11$^{NLSII^-}$ transfectants exhibited this pattern, we suggest that at least in some cases, NLSIII can substitute for NLSII and act in concert with NLSI, although with a reduced efficiency.

To further confirm the conclusion that nuclear homing of p53 protein is dominated by both NLSI and NLSII, we

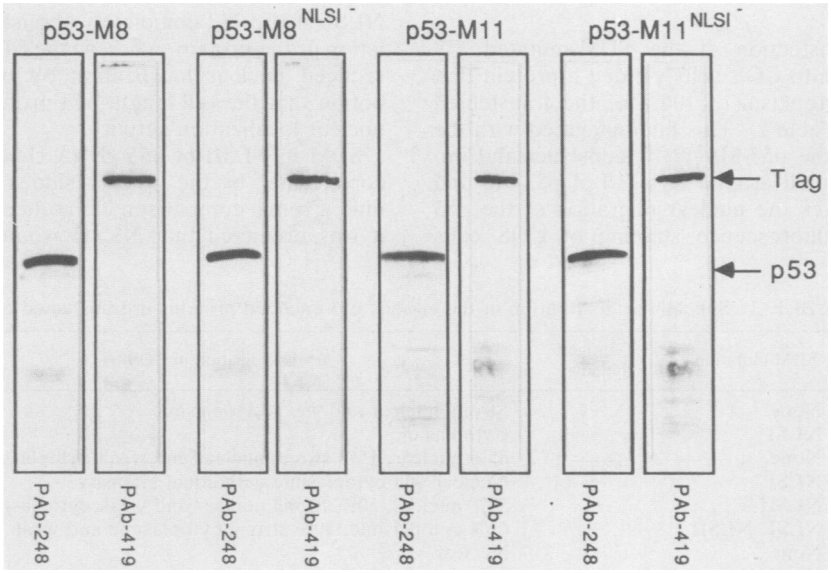

FIG. 5. Expression of NLS-modified p53 cDNA clones in COS cells. Cells were transfected with the constructs indicated above the lanes. Equal amounts of cell lysates were used to detect p53 expression by Western blot analysis, using the anti-p53 monoclonal antibody PAb-248. Endogenous SV40 large T antigen (T Ag) levels were determined by using the anti-large T monoclonal antibody PAb-419.

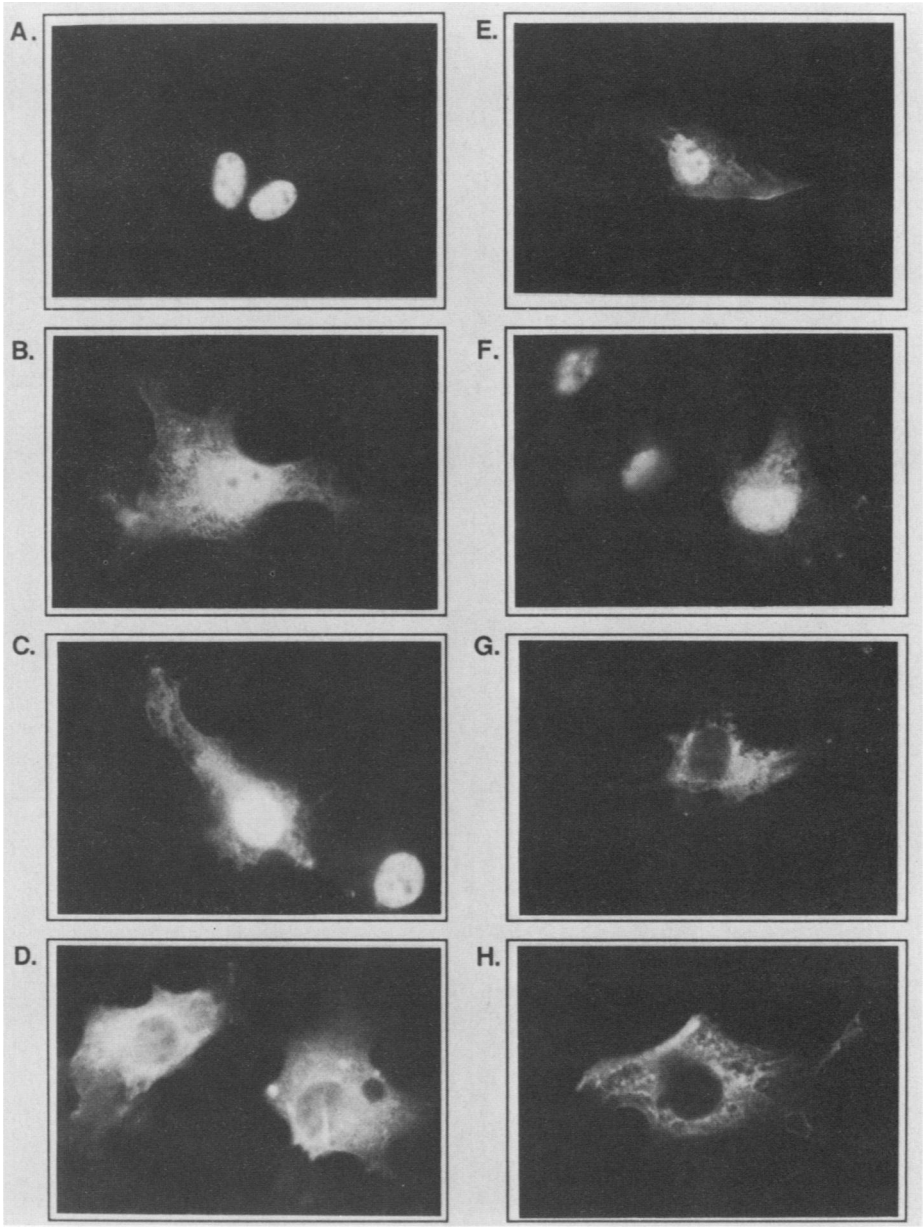

FIG. 6. Alterations in the subcellular localization of NLSI- and NLSII-modified p53 proteins. COS cells were transfected with the various p53 cDNA clones constructed in the pSVL vector. At 24 to 36 h after transfection, the cells were fixed and processed for indirect immunofluorescence staining with different p53-specific monoclonal antibodies. (A) Cells transfected with p53-M11 and stained with PAb-248; (B) cells transfected with p53-M11$^{NLSI^-}$ and stained with PAb-248; (C) cells transfected with p53-M11$^{NLSII^-}$ and stained with PAb-248; (D) cells transfected with p53-M11$^{NLSI^-,NLSII^-}$ and stained with PAb-248; (E and F) cells transfected with p53-M8 and stained with PAb-242 (E) and PAb-248 (F); (G and H) cells transfected with p53-M8$^{NLSI^-}$ and stained with PAb-242 (G) and PAb-248 (H).

constructed a p53-M11-derived plasmid coding for a full-length p53 protein that was mutated at both NLS sequences. To that end, we replaced the intact NLSII of p53-M11$^{NLSI^-}$ with a corresponding DNA fragment of p53-M11$^{NLSII^-}$ which carried the mutation in NLSII. The structure of the resulting construct, p53-M11$^{NLSI^-,NLSII^-}$ was confirmed by DNA sequencing. Western blot analysis showed that the construct coded for the correct p53 protein (data not shown). Staining of the transfected cells (Fig. 6D; Table 1) revealed that in more than 50% of the cells, p53-M11$^{NLSI^-,NLSII^-}$ encoded a protein that accumulated in the cytoplasmic compartment. Thus, NLSI and NLSII act in concert to direct the nuclear migration of p53. In the rest of the cells, p53 was found mainly in the cytoplasm, but a significant nuclear staining was also evident. Since the staining pattern of these cells resembled very much that of p53-M11$^{NLSI^-}$ (Fig. 6B), it appears that the putative NLSIII can direct some of the mutated protein into the nucleus.

An analysis of the wild-type p53-cD showed that in all transfected cells containing SDM mutations either in NLSI or in both NLSI and NLSII, there was a rather high incidence of nuclear staining (Fig. 7; Table 1). COS cells transfected with p53-cD$^{NLSI^-}$ exhibited an exclusive nuclear staining in 70 to 80% of the cells (Fig. 7C). The rest of the

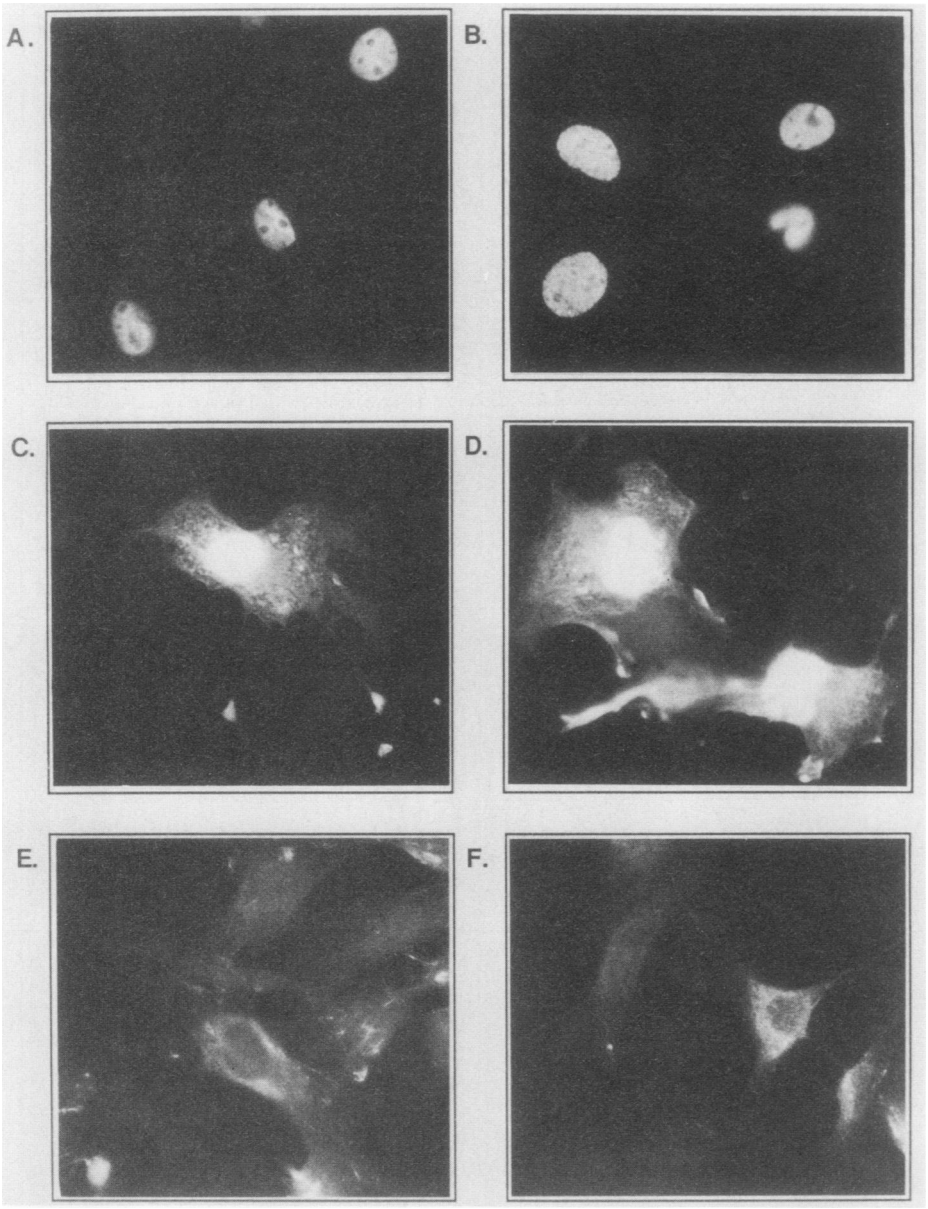

FIG. 7. Alterations in the subcellular localization of NLS-modified wild-type p53 protein. COS cells or primary REF were transfected with the various p53-cD derived cDNA clones in the pSVL vector or with the pSVL vector alone. At 24 to 36 h after transfection, the cells were fixed and processed for indirect immunofluorescence staining with PAb-419 monoclonal antibodies directed against the SV40 large T antigen or with PAb-248 monoclonal antibodies directed against the p53 protein. (A) COS cells transfected with pSVL alone and stained with PAb-419; (B) COS cells transfected with p53-cD and stained with PAb-248; (C) COS cells transfected with p53-cD$^{NLSI^-}$ and stained with PAb-248; (D) COS cells transfected with p53-cD$^{NLSI^-,NLSII^-}$ and stained with PAb-248; (E) primary REF transfected with p53-cd$^{NLSI^-,NLSII^-}$ and stained with PAb-248; (F) primary REF transfected with p53-cD$^{NLSI^-,NLSII^-}$ and stained with PAb-242.

cells displayed, in addition, a clear but weaker cytoplasmic staining. In the cells transfected with the doubly SDM-mutated p53-cD$^{NLSI^-,NLSII^-}$, about 60% of the cells showed an exclusive nuclear staining. In the rest of the cells, the strong nuclear staining was accompanied by different levels of cytoplasmic staining (Fig. 7D). This nuclear staining observed in NLS-deprived p53-cD differed significantly from the equivalent in NLS-deprived mutant p53. We therefore suggest that the large T antigen, which is expressed in COS cells and forms complexes with the wild-type p53, may play an important role in the nuclear localization of this protein. The mutant p53 proteins, which do not form a complex with the large T antigen, are totally dependent on the presence of their inherent NLS sequences for nuclear localization.

To test this hypothesis, we repeated these experiments in primary REF that are free of transforming viral proteins. Expression of the transfected exogenous p53 protein was recognized by the murine-specific anti-p53 monoclonal antibodies PAb-242 and PAb-248. Figures 7E and F shows typical patterns of p53 subcellular distribution of REF transfected with SDM-mutated p53-cD$^{NLSI^-,NLSII^-}$ and stained with PAb-242 and PAb-248, respectively. In general, we found that the subcellular distributions of the wild-type and mutant p53 proteins in primary REF were similar. In both,

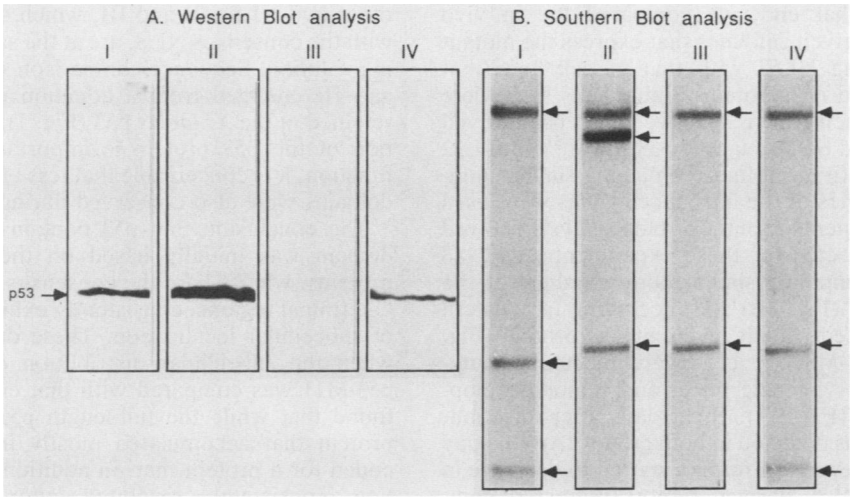

FIG. 8. Analysis of L12-derived cell lines transfected with p53-M8 and p53-M8$^{NLSI^-}$. Western blot analysis was used to detect the expression of p53 in the L12-derived clones (A). A total of 5×10^7 exponentially growing cells were lysed and processed for immunoblotting with monoclonal antibody PAb-248 as described in Materials and Methods. The integrated DNA sequences were detected by Southern blot analysis (B). Genomic DNA was prepared from 5×10^7 exponentially growing cells and digested to completion with *Bam*HI. The DNA was fractionated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. Specific DNA sequences were detected by hybridization to a nick-translated p53-M8 cDNA fragment. The top and middle arrows designate the endogenous p53 fragments of L12; the bottom arrow designates the integrated p53 cDNA. The additional arrow in lane II designates the integrated genomic fragment of the L20-8D6 cell line.

nuclear localization was totally dependent on the presence of active NLS domains (Table 1).

NLS is required for p53 protein to enhance the transformation of L12 p53-nonproducer cells. The natural migration of the p53 protein into the cell nucleus strongly suggests that its nuclear localization is important for the biological activity of the protein. Although the exact function of p53 is unknown, several bioassays to measure its activity in malignant transformation have been developed (14, 23, 40, 56). We have used cells of a p53-nonproducer Abelson murine leukemia virus-transformed lymphoid cell line (L12) which develop into regressor tumors when injected into syngeneic mice (56). p53 expression was abolished in L12 cells because the gene was rearranged by integration of a Moloney viral particle into the first p53 intron (54, 57). Transfection of mutant genomic p53 clones into L12 cells restored the expression of p53 and changed the phenotype of the L12 cells to cells that are lethal for the host (56).

We tested whether nuclear localization of the mutant p53 protein is essential in enhancing the process of malignant

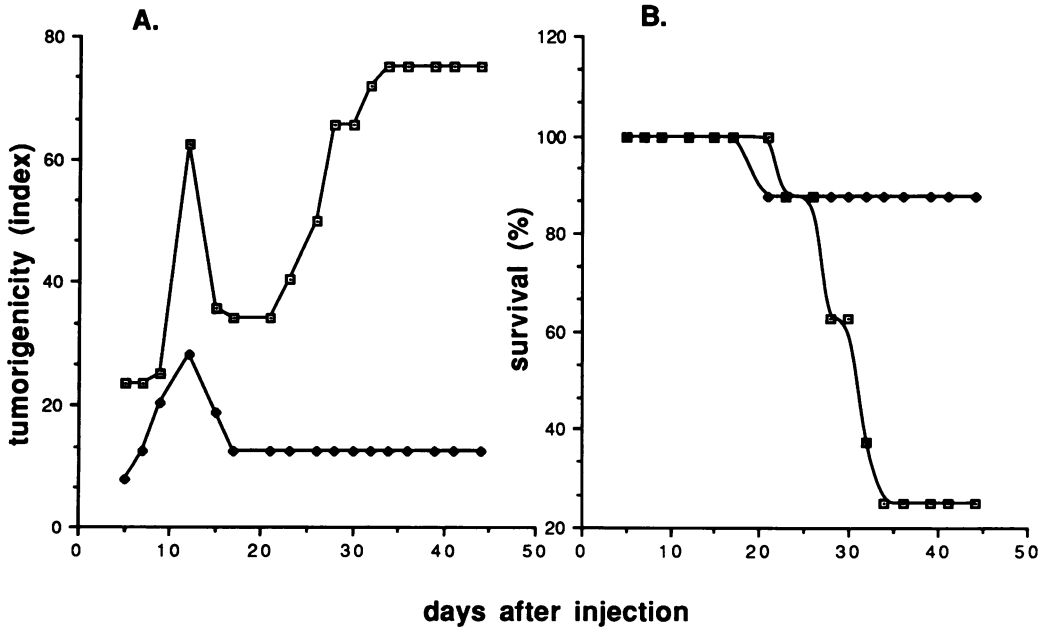

FIG. 9. Effect of nuclear localization on tumorigenicity of L12 cells in syngeneic mice. C57L/J mice were injected subcutaneously with L12(p53-M8) cells (□) or L12(p53-M8$^{NLSI^-}$) cells (■——■). Tumor development (A) and survival (B) are presented as a function of time after injection. See Materials and Methods for details.

transformation. To that end, we compared the in vivo malignancy of L12-derived cell lines that express the mutant p53 protein containing NLSI with that of cell lines that express NLS-deprived p53 protein. Stable cells lines were established by introducing the respective pSVL p53-derived constructs p53-M8 and SDM-mutated p53-M8$^{NLSI^-}$ into L12 cells. Two representative cell lines containing similar numbers of integrated copies of the introduced genes were used in the in vivo experiments. Southern blot analysis showed that the cell lines selected for these experiments, L12-M8 and L12-M8$^{NLSI^-}$, contained similar copy numbers of the integrated p53 cDNA (Fig. 8B) and, according to Western blot analysis, expressed similar levels of p53 protein (Fig. 8A). L12-M8 and L12-M8$^{NLSI^-}$ cells were injected subcutaneously into C57L/J syngeneic mice, and tumor development was monitored (Fig. 9A). Eight days after injection, local tumor growth was detected in both groups. Around day 12 postinjection, however, the tumors started to decrease in size. While L12-M8$^{NLSI^-}$-derived tumors regressed completely, L12-M8-derived tumors developed further, and all of the tumor-bearing animals eventually died. The lethality curve for the experiment is presented in Fig. 9B.

The results show that the p53-M8 cDNA, which contains only the principal p53 homing domain, NLSI, functions as an oncogene in the L12 system and that a single-base substitution in this domain concomitantly abolishes its nuclear migration and causes a significant reduction in the ability of p53 to transform L12 cells. We conclude, therefore, that it is most likely that the nuclear localization of the p53 protein is essential for the mutant protein to exhibit its oncogenic activity in the L12 system.

DISCUSSION

The observation that p53 is frequently detected in the cell nucleus suggests that nuclear localization of this protein is most likely important for p53 activity. In agreement with recent reports, we found that the p53 protein migrates into the nucleus by means of NLS sequences inherent in the primary DNA sequence of the protein. NLSI is the principal active domain. Indeed, Dang and Lee (9) showed that fusion of human p53 NLSI with pyruvate kinase sequences yielded a protein that localized within the cell nucleus. Staining of transfected cells with anti-pyruvate kinase antibodies indicated specific nuclear distribution of the transfected protein (9). In this study, we mutated the NLSI of mouse p53 and found that NLSI-deprived p53 exhibited an altered subcellular localization. On the basis of studies with the large T antigen (24, 25), we predicted that replacement of the middle Lys residue of NLSI with a Thr would perturb the specific basic core formed by the three consecutive lysine residues. As a result of this manipulation, the protein encoded by SDM-mutated p53-M8$^{NLSI^-}$ failed to enter the nucleus of transfected COS cells and instead accumulated in the cytoplasmic compartment, indicating that the central Lys of NLSI is a critical element in the activity of this domain. A similar approach was recently taken by Addison et al. (1), who showed that replacement of all three Lys residues of NLSI by alanines interfered with the nuclear homing of human p53.

The experiments presented here show that p53 contains NLS domains other than NLSI that actively mediate nuclear localization of this protein. The most N-terminal signal, NLSI, is highly conserved in genetically diverged species and shares perfect homology with the consensus NLS sequences found in other nuclear proteins (7, 24, 43, 58). The other two NLSs, II and III, which share a lower homology with the consensus NLS, are at the same time less conserved in evolution. Sequence comparison suggests that domains II and III emerged from a common primordial NLS that is retained in the *X. laevis* p53 (Fig. 1). Since nuclear localization of the p53 protein is important for its physiological function, it is conceivable that less efficient, alternative NLS domains were also conserved during evolution.

The conclusion that p53 contains a second active NLS domain was initially based on the observation that p53 proteins, which share the consensus NLSI but vary in other C-terminal regions, consistently exhibited a different pattern of subcellular localization. These differences were evident when the subcellular distribution of protein encoded by p53-M11 was compared with that encoded by p53-M8. We found that while the full-length p53-M11 coded for a p53 protein that accumulated mostly in the nucleus, p53-M8 coded for a protein that, in addition to its nuclear localization, consistently exhibited cytoplasmic staining. Since p53-M8 cDNA was truncated as the result of an alternative splicing at the C terminus (3), we predicted that in addition to NLSI shared by the two cDNAs, the C-terminal part of the p53-M11-encoded protein contained additional NLS sequences. This assumption was further confirmed by the observation that while p53-M8$^{NLSI^-}$-encoded protein showed an exclusive cytoplasmic distribution, p53-M11$^{NLSI^-}$ gave rise to a p53 protein that was equally distributed between the cytoplasm and the nucleus. By means of SDM, we showed that the basic sequence of Lys–Thr–Lys–Lys-369, situated downstream to NLSI, is a second NLS. NLSII shares lower homology with the consensus NLS and is therefore expected to be less efficient. This may explain the observation that p53-M11$^{NLSII^-}$-encoded protein migrates into the nucleus with an efficiency resembling that of the p53-M8-encoded protein.

The existence of the third NLS was mainly deduced from the observation that the p53 protein deprived of both NLSI and NLSII, encoded by a full-length cDNA, still showed a certain frequency of nuclear staining. Conversely, p53 cDNA clones with deletion of the entire 3′ end consistently showed cytoplasmic staining. Sequence analysis indicated the presence of Ser–Arg-376–His–Lys–Lys–Thr that was suggested to be NLSIII. This is a basic sequence that resides in the carboxy terminus of the full-length protein, downstream of NLSII. Judging by the intensity and frequency of p53 subcellular localization, it is conceivable that although NLSI is the principal NLS of p53, the two additional NLSs function in an additive manner. The fact that none of the individual NLSs can give rise to a p53 protein that is exclusively nuclear, as can the full-length protein containing all three domains, strongly suggests that the in vivo nuclear localization of p53 requires the activity of all three domains in the cell.

It should be noted that other nuclear proteins have also been found to contain more than one NLS. For example, the nuclear localization of adenovirus DNA-binding protein has been shown to require two nuclear signals (31, 36). Likewise, two signals mediating the hormone-dependent localization of the glucocorticoid receptor were detected (41). The existence of several domains that apparently function in a similar way strongly suggests that nuclear localization is a fundamental feature in the activity of these proteins, which is why the cells have retained more than one such structural domain.

Recent studies have shown that wild-type and mutant p53 genes differ in their biological activities. While the wild type

functions as a suppressor gene in the manner of a growth arrest gene (13, 15, 30), the mutant enhances malignant transformation (12, 14, 23, 40, 56, 57). It was therefore of interest to find out whether the two vary in subcellular localization.

Sequence comparison of wild-type and mutant p53 showed that the two protein categories contain similar NLS sequences; they are therefore expected to migrate into the nucleus by essentially similar mechanisms. However, we found that the actual nuclear localizations of wild-type and mutant p53 proteins differ in various cell types. When p53 plasmids were transfected into primary REF, the patterns of nuclear localization of mutant and wild-type proteins were identical. In both, nuclear localization was dominated by the presence of the NLS domains. Proteins that contained intact NLS domains efficiently localized into the nucleus, whereas those deprived of these signals accumulated within the cytoplasm. In COS cells transformed by SV40, nuclear localization of p53 seemed to be controlled by additional factors that overrode the dependence of nuclear localization on the NLS sequences. Indeed, we found that the NLS-deprived wild-type p53 molecule may migrate into the nucleus of COS cells, most likely by forming a complex with another nuclear protein, the large T antigen. The fact that wild-type and mutant p53 proteins differentially complex with viral (30, 32, 34, 46, 52) or cellular proteins, as do, for example, the major heat shock proteins (16, 21, 42), may have a significant impact on the ultimate compartmentalization of p53 in the cell. The large T antigen may modify the p53 nuclear localization by means of at least two mechanisms. In one, the large T antigen, while still in the cytoplasm, forms a complex with the NLS-deprived wild-type p53 and the complex then migrates into the nucleus by utilizing the NLS sequences of the large T antigen. The alternative mechanism consists of shuttling of nuclear proteins in and out of the nucleus (5, 49). An equilibrium between nuclear and cytoplasmic p53 is achieved, which is dependent on the number and efficiency of the NLSs. When the large T antigen is present in the nucleus, the equilibrium diverges toward the nuclear accumulation of the wild-type p53. Mutant p53 proteins, which lack the ability to complex with the large T antigen, localize in the nucleus according to the number of effective NLSs in their primary sequences. A similar situation, in which one nuclear protein facilitates the nuclear localization of a second protein, was found in the case of adenovirus DNA polymerase: the nuclear transport of adenovirus DNA polymerase is facilitated by interaction with preterminal protein, irrespective of the presence of its nuclear localization signal (31).

Sequence variations between wild-type and mutant p53 proteins were shown to modify the biological activity of the protein and its capacity to bind cellular or viral proteins. Although not found in the NLS domain, these mutations may eventually affect the subcellular localization of p53. The actual nuclear homing of proteins may be modified by interactions with other proteins expressed in the cell. It can therefore be assumed that the cellular milieu in general may significantly affect the normal pattern of subcellular localization of proteins. We would like to advance the idea that an alteration in the subcellular localization, occurring in tumor cells because of complex formation with tumor-specific proteins, may play a key role in the activity of p53 in malignant transformation.

To address this issue more directly, we tested the significance of nuclear localization of p53 with respect to the capacity of mutant p53 to enhance malignant transformation. To that end, we compared the ability of intact and NLS-deprived mutant p53 to enhance the transformation of partially transformed L12 cells. The L12 cells, which do not express p53 protein because of a viral integration into the first intron of the genomic p53, form local tumors that eventually regress in syngeneic mice (54, 57). Previously, we had found that restoration of p53 expression in the L12 line led to the acquisition of a fully malignant phenotype which resulted in the development of lethal tumors in mice (56). p53-M8, containing a single NLS, which was shown to function as a dominant oncogene in other experimental systems (12), was selected for these experiments. We expected that modification of the single NLS of this protein would directly answer the question of the significance of nuclear localization for the transforming activity of the protein. Comparison between p53-M8$^{NLSI^-}$ and p53-M8 showed that the intact p53-M8 caused malignant transformation of L12 cells, whereas the NLSI-defective mutant failed to fully transform the cells. The conclusion was that the nuclear localization of p53 is essential for its transforming activity. These results provided further support for the hypothesis that the p53 gene can act as a dominant oncogene in cells lacking any wild-type p53 and directly indicate that the in vivo malignant transforming activity mediated by mutated p53 protein depends on its nuclear localization.

To conclude, the nuclear localization of p53 is dependent on the presence of three NLSs in the primary amino acid sequence of the protein, and the oncogenic activity of p53 protein is dependent on its nuclear localization. The nuclear localization signals are therefore important factors in the physiological activity of p53.

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